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Hydroxycinnamic Acid Amide Derivatives, Phenolic Compounds and Antioxidant Activities of Extracts of Pollen Samples from Southeast Brazil

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ABSTRACT: Seven bee pollen samples (C1—C7) with different palynological sources were harvested from Pindamonhangaba municipality (Southeast Brazil). Methanol extracts of untreated samples (control), samples frozen at −18 °C and samples frozen and then dried were analyzed by HPLC/PAD/ESI/MS/MS. Flavonoid diglycosides of quercetin, kaempferol, isorhamnetin and patuletin were detected, together with hydroxycinnamic acid amide derivatives, such as *N*′,*N*″,*N*‴-tris-*p*-feruloylspermidine and *N*′,*N*″,*N*‴-tris-*p*-coumaroylspermidine. Distinct phenolic profiles characterized the analyzed samples, but no differences were noted as resulting from different treatments. Total phenolic contents determined with the Folin—Ciocalteau reagent ranged from 1.7 to 2.2%. Antioxidant activities above 75%, based on the DPPH method, were observed for all extracts, not correlated with total phenolic contents. Among samples from the same origin, those frozen were more active than samples untreated (control), and the samples frozen and then dried were the most active.

KEYWORDS: *Apis mellifera*, bee pollen, flavonoids diglycosides, *N*′,*N*″,*N*‴-tris-*p*-feruloylspermidine

INTRODUCTION

Pollen is a fine powder formed by microscopic grains containing the plant reproductive male cell. Pollen grains contain a broad variety of substances. These include an extremely stable biopolymer, the sporopollenin of their outer layer (so far with composition and structure scarcely known), in addition to lignin, pectin, proteins, lipids, carbohydrates, nucleic acids, pigments, flavonoids and carotenoids.[1] Wind, water, bees and other insects transfer pollen from stamens to the flower stigmata. Honey bees collect pollen by adding sugars from nectar to bind the grains together and then take them to the colony, packing the pollen masses onto hairs in the corbiculae of the hind legs. Pollen is placed in honeycombs, mixed with honey. The role of pollen in the sustenance of the bee colony cannot be overestimated; bees consume pollen in their own diet and use it to feed the larvae.[2] Bee pollen of commerce stems from *Apis mellifera*, but many other social species of bees also use flower pollen, such as the stingless bee *Melipona subnitida* ("jandaira"), native in Northeast Brazil.[2]

Bee pollen is a mixture of material from different plant species. This apicultural product has been used in the human diet for many centuries, due to its nutritional value.[3] It contains flower pollen mixed with nectar and bee secretions and is rich in sugars, proteins, lipids, vitamins, carbohydrates and flavonoids.[3] A relationship has been shown between the vitamin content and the plant source of pollen.[4] Daily ingestion of pollen has been recommended, because it regulates the intestinal functions, has beneficial effects on the cardiovascular system, skin and vision, and has antibiotic, anticancer, antidiarrheic and antioxidant activities.[2−9]

Recent papers have reported the chemical composition of pollen.[2,4,10,11] Usually, it contains flavonoid glycosides and hydroxycinnamic acid derivatives. Flavonol glycosides, particularly of kaempferol, quercetin and isorhamnetin, have a broad spectrum of biological activity.[2,5] Some pollen contains also flavonoid aglycons, such as luteolin, quercetin and myricetin.[3] High contents of rutin and myricetin were found in bee pollen produced in Southern Brazil.[6] A high diversity of glycosides of flavonols and flavones, together with other phenolic substances, has been reported in bee pollen.[5,7−9] Hydroxycinnamic acid amides, commonly found in floral parts, have been isolated and identified in pollen.[10,11] Triacylated spermidine conjugates were found as major constituents of pollen from oak species; pollen from Betulaceae and Juglandaceae contained diacylated spermidine conjugates.[10] Several functions have been assigned to

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Table 1. Plant Species Contributing Pollen to Samples C1−C7 Collected from Bee Hives in the Municipality of Pindamonhagaba (Southeast Brazil)[a]

plant species or pollen type	C1	C2	C3	C4	C5	C6	C7
Antigonum leptosus	0.0	0.0	0.0	0.0	0.0	0.2	0.0
Anadenanthera sp.	0.0	23.0	4.4	18.4	18.4	49.5	42.9
Cecropia sp.	0.4	0.0	0.0	0.0	0.0	0.0	0.0
Didymopanax sp.	21.4	11.0	0.0	0.0	0.0	0.1	0.0
Eucalyptus sp.	1.1	13.0	0.0	39.1	0.0	0.6	0.1
Eupatorium sp.	0.0	0.0	22.8	0.0	0.0	0.0	0.1
Macroptilium sp.	0.0	0.0	0.0	0.0	0.0	0.0	6.4
Myrcia sp.	13.6	31.9	45.3	33.3	53.8	14.3	28.5
Philodendron sp.	0.0	0.0	0.0	0.0	0.0	1.5	0.0
Raphanus sp.	0.0	0.0	0.0	0.0	0.0	0.0	0.1
Alismataceae type	31.4	9.6	13.6	0.0	0.0	0.0	0.0
Arecaceae (palm) type	32.1	11.5	13.9	9.2	27.8	33.9	21.1
Poaceae (grass) type	0.0	0.0	0.0	0.0	0.0	0.1	0.0
unidentified	0.0	0.0	0.0	0.0	0.0	0.0	0.8

[a] Values represent percent means from triplicate determinations. Adapted from Oliveira et al.[4]

hydroxycinnamic acid amides, including protection against fungi, bacteria, viruses and insects, as well as regulation of growth, cell multiplication, floral induction and flower formation.[11]

Active oxygen free radicals have been implicated in a variety of health problems. Both normal metabolism and exogenous factors (UV light, carcinogens) influence free radical production.[12] They may bring about breakdown of vital cellular components, such as coenzymes, neurotransmitters, nucleic acids, proteins, lipids and carbohydrates. It is believed that the ingestion of exogenous antioxidants may improve the natural capacity of living cells to overcome the activity of these oxidative free radicals.[13] Phenolic hydroxyls are highly correlated with strong antioxidant activity,[14,15] and flavonoids are effective antioxidants.[16] Therefore, pollen is considered an important source of natural antioxidants.[8,17] Rapid and efficient methods to evaluate antioxidant activity have been proposed. The method of the DPPH free radical (1,1-diphenyl-2-picrylhydrazyl) probably has been the most used, due to the stability of the reagent, its high solubility in alcohol and intense absorbance in the visible region.[8,18]

Methods for chemical analysis of bee pollen are needed, in view of the growing interest in its therapeutic properties. In this regard, the analysis of phenolic compounds deserves attention as an aid for evaluation of chemical composition and consequently the quality of pollen. The aim of the present work was to analyze the chemical composition of seven pollen samples (C1−C7) from Southeast Brazil, collected during 21 days, and to evaluate their antioxidant activity. Evaluation of the effects of different treatments on the antioxidant activity and chemical composition of the pollen samples was also an objective of the present work.

MATERIALS AND METHODS

Pollen Sources. Floral pollen used in this study was obtained from pellets collected at Agência Paulista de Tecnologia dos Agronegócios (APTA), Pindamonhangaba municipality (22° 57′S, 45° 27′W, 560 m, São Paulo State, Brazil), involving seven samples (C1−C7). Table 1 lists sources of pollen constituting the samples C1−C7. Details of palynological analyses are described elsewhere.[4]

The beehives were weekly visited for monitoring parameters such as population, proportion of sucklings, possibility of entrance of pollen, and presence of the queen bee, as well as provision of nongreased soy flour-based protein supplement[19] and food supplement (60% sugar syrup).[4]

Collection and Treatment of Bee Pollen. Bee pollen samples were harvested from containers installed in beehives of Africanized bees *Apis mellifera*. The samples were obtained with 72 h intervals during 21 days, comprising seven collections (C1−C7). All samples were divided in three parts, each undergoing a distinct treatment: (1) unaltered (control); (2) frozen at −18 °C during 48 h; (3) frozen at −18 °C during 48 h and then dehydrated. In the latter treatment, the frozen samples were dried in a tray with air circulation at 40−42 °C, until moisture was reduced to 4%. Extracts were obtained from 1.0 g of pollen, upon treatment with 75.0 mL of 70% methanol during 45 min in the dark, at room temperature. The extracts were diluted to make up 100 mL with methanol 70% for chemical analyses.

Hydrolysis of Methanol Extracts of Pollen. To release flavonoid aglycons, a hydrolysis reaction was carried out.[16] Methanol extracts of bee pollen (20.0 mL) were treated with 20.0 mL of 10% H_2SO_4 at boiling temperature for 1 h. After cooling, the supernatant was neutralized with saturated aqueous sodium carbonate and filtered under reduced pressure. The solution was evaporated to dryness under vacuum and redissolved in 3.0 mL of methanol:water (1:1, v/v) for HPLC/PAD analyses.

Total Phenolic Content. Total phenols of C1−C7 of untreated samples were determined by the Folin−Ciocalteau method,[16] using 0.5 mL of methanol extract and gallic acid as reference.

DPPH Antioxidant Activity. The antioxidant activity of the bee pollen samples (C1−C7) undergoing the three treatments was determined by the radical scavenging activity method of the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH),[20] using a 20.0 μg/mL of methanol solution of DPPH prepared to absorb in the range 0.7−0.8 at 517 nm. An aliquot of 0.1 mL from each methanol extract (1 mg/mL) was added to 3.9 mL of the DPPH methanol solution. Thus, the final extract concentration in contact with DPPH was 25 μg/mL. A blank was prepared with 0.1 mL of methanol and 3.9 mL of DPPH solution. BHT at the final concentration 5 μg/mL was used as positive control. After 20 min at room temperature in the dark, the absorbance of all solutions was determined at 517 nm. The radical scavenging activity was calculated as follows: $I\% = [(Abs_0 - Abs_i)/Abs_0] \times 100$, where Abs_0 is the absorbance of the blank and Abs_i the absorbance of the samples. The assays were carried out in triplicate. All pollen extracts and DPPH solutions were freshly prepared.

HPLC/PAD/ESI/MS/MS Analyses. Extracts of C1−C7 (100.0 μL) were dissolved in 3.0 mL of methanol:water (20:80, v/v) and filtered through a 0.45 μm filter (German Sciences, Tokyo, Japan) prior to injection of 30.0 μL into the HPLC/MS/MS equipment. A SPD-M10AVP Shimadzu chromatograph equipped with a photodiode array detector (PAD) was coupled to a mass spectrometer Esquire 3000 Plus, Bruker Daltonics, via an electrospray ionization (ESI) source. Mobile phases used were A (0.1% aq HOAc) and B (methanol). A reverse phase C18 Zorbax 5B-RP-18 (Hewlett-Packard) column (4.6 × 250 mm, 5 μm) was used, with the following gradient: 0 min, 20% B in A; 10 min, 30% B in A; 20 min, 50% B in A; 30 min, 70% B in A; 40 min, 90% B in A; 45 min, 40% B in A; and return to the initial condition (20% B) to re-equilibrate the column. The flow rate was kept constant at 0.5 mL/min, and the temperature of the column was maintained at 28 °C. PAD worked at intervals of 200−600 nm, and the optical density of the eluate was continuously monitored at 270 and 350 nm. Electrospray ionization was performed using an ion source voltage of −40 V and a capillary offset voltage of 4500 V. Nebulization was aided with a coaxial nitrogen sheath gas provided at 27 psi. Desolvation was assisted using a counter current nitrogen flow set at flux 7.0 L/min and a capillary temperature of 325 °C. The flux of LC/MS was 100 μL/min. ESI/MS parameters, such

Table 2. Retention Time (t_R, HPLC), UV and Mass Spectral Data of Compounds Detected in Methanol Extracts of Pollen Samples C−C7[a], and Respective Proposed Identification

compound	t_R (min)	λ_{max} (nm)	$(M + H)^+$/ $(M - H)^-$ (m/z)	MS/MS positive ion mode (m/z)	proposed identification
1	5.1	310	685.0/683.0		rosmarinic acid dihexoside derivative
2	24.2	254, 265 sh, 356	627.2/625.0	481.0 (60%); 319.0 (100%)	myricetin-3-*O*-rhamnosyl-glucoside
3	24.8	255, 264 sh, 355	435.0/433.2	303.0 (100%)	quercetin-3-*O*-arabinoside
4	25.0	253, 265 sh, 357	641.2/639.0	479.0 (60%); 317.0 (100%)	isorhamnetin-3-*O*-diglucoside
5	26.0		595.0/593.2	449.0 (70%), 287.0 (100%)	kaempferol-3-*O*- rhamnosyl-glucoside
6	26.4	254, 265 sh, 356	771.2/769.0	625.1 (90%), 479.1 (50%), 317.1 (100%)	isorhamnetin-3-*O*-(2′′,3′′-dirhamnosyl)glucoside
7	26.8	256, 359	611.1/609.1	303.1 (100%)	rutin
8	27.0	254, 265 sh, 360	625.2/623.0	479.0 (50%); 317.0 (100%)	isorhamnetin-3-*O*- rhamnosyl-glucoside
9	28.0	255, 360	449.0/447.0	287.0 (100%)	kaempferol-3-O-glucoside
10	28.6		641.2/639.0	495.0 (40%); 333.0 (100%)	patuletin-3-*O*- rhamnosylglucoside
11	30.1		449.2/447.1	303.1 (100%)	quercitrin
12	30.8	380	381.4/379.5	261.0 (20%); 235.2 (100%); 147.2 (40%)	chalcone
13	31.4	255, 350	761.3/759.1	615.3 (100%); 381.1 (20%); 287.0 (40%)	kaempferol-7-*O*-rhamnosyl-3-*O*-galloyl glucuronide
14	32.5	300	584.3/582.1	438.2 (100%); 420.2 (80%)	*N′*,*N′′*,*N′′′*-tris-*p*-coumaroylspermidine
15	34.6	300 sh, 330	674.3/672.2	498.2 (70%); 480.3 (100%)	*N′*,*N′′*,*N′′′*-tris-*p*-feruloylspermidine

[a] See Table 1.

as nature and flow rate of the sheath liquid, nebulizer pressure, drying gas flow rate and temperature, were optimized. MS data were acquired in the positive and negative ion modes. The full scan mass acquisition was performed by scanning in the range m/z 100−900. In order to confirm the experimental data obtained, the precision for each sample was evaluated for intraday (n = 3) and interday (n = 6) repeatability. Compounds were identified by comparison of corresponding UV chromatogram peaks and ESI/MS/MS spectra with MS database (literature) and comparison with standards.

The products of hydrolysis were analyzed by HPLC/PAD, and the aglycons released were identified by comparison with standards. Rutin, kaempferol, quercetin and isorhamnetin were used as standards to aid in the identification.

Statistical Analyses. Statistical analyses were carried out using the SAS program (Statistical Analysis System, 1990).

RESULTS AND DISCUSSION

Plant Sources of Flower Pollen. Bees are highly selective pollen gatherers. Bee pollens have been shown to comprise flower pollen from few of the plant species potentially available in the surroundings.[1,3] Few species contributed with pollen to pellets of local bees (Table 1). Predominant pollen in C1 stemmed from Alismataceae and Arecaceae (monocotyledons); in C2, from *Anadenanthera* and *Myrcia*; in C3, from *Eupatorium* and *Myrcia*; in C4, from *Eucalyptus* and *Myrcia*; in C5, from Arecaceae and *Myrcia*; in C6 from *Anadenanthera* and Arecaceae; and in C7, from *Anadenanthera*, Arecaceae and *Myrcia*. Some species seem to be abundant sources (*Anadenanthera*, palms, *Eucalyptus*, *Myrcia*), while other species contribute very little (*Antigonum*, *Cecropia*, *Philodendron*, grasses). In spite of the high diversity of plants in the region, pollen from no more than thirteen plant species has been detected.

Bee Pollen Chemical Constituents. Nectar, pollen and propolis are abundant sources of phenolic compounds often incorporated into honey.[21] The products of the hydrolysis of methanol extracts of bee pollen yielded flavonol aglycons, such as quercetin, kaempferol and isorhamnetin, which were identified chromatographically by comparison with standards. Retention times (t_R), wavelengths of maximum absorbance (λ_{max}), protonated/deprotonated molecular ions ($[M + H]^+$/ $[M - H]^-$) and major fragment ions (MS/MS in the positive ion mode) are listed in Table 2.

The combination of both ionization modes (positive and negative) gave support to the identification of quasi-molecular ions. PAD is important for identification of phenolic constituents of plant extracts, being useful to distinguish among flavonoids and cinnamic acid derivatives. Chalcones with hydroxyl at position 4 of the B ring have characteristically high band *I* values (380.0 nm, Table 2).[16] UV data (λ_{max} 254, 265 sh, and 356 nm) of all flavonoids found were consistent with flavonol glycosides with substitution at the 3-hydroxyl. Pollen samples C1−C7 contain flavonol glycosides, mainly derivatives of isorhamnetin, quercetin and kaempferol, an unknown chalcone, together with hydroxycinnamic acid amide derivatives, such as *N′*,*N′′*,*N′′′*-tris-*p*-feruloylspermidine and *N′*,*N′′*,*N′′′*-tris-*p*-coumaroylspermidine.

Compound **1** (Table 2) showed UV maximum absorption (λ_{max}) at 310 nm and a deprotonated molecular ion at m/z 683.0. MS/MS spectrum in the negative ion mode showed a base peak at m/z 341, indicating the presence of a caffeoyl hexoside moiety, and another fragment at m/z 179 (50%), corresponding to a caffeic acid moiety. Probably, this compound is formed by two main moieties of hydrocaffeic and caffeic acid dihexosides. Rosmarinic acid is an ester of caffeic acid with 3,4-dihydroxyphenyl lactic acid. Thus, compound **1** may be a rosmarinic acid dihexoside derivative. Martinoside, a compound with such characteristics, was characterized in lemon verbena.[22]

Losses of fragments with 162 amu (hexose) and 146 amu (deoxyhexose) were observed in the MS/MS spectra of flavonoids in the positive ion mode. Such fragments were assumed to correspond to glucosyl and rhamnosyl moieties, respectively, corresponding to the most frequent sugars in flavonoid glycosides. The most common disaccharides found in association with flavonoids are rutinose and neohesperidose. Distinction between these two types of disaccharides is possible on the basis of the different relative abundances of the Y_1^+ ion, corresponding to $[M + H - Rha]^+$ and the Y_0^+ ion attributed to the protonated

aglycon.[23,24] Compound **2** (Table 2) showed a protonated molecular ion at m/z 627.2 $(C_{27}H_{31}O_{17})^+$. The MS/MS spectrum in positive ion mode showed a fragment ion at m/z 481.0 (60%) (Table 2), suggesting the loss of a rhamnosyl moiety, and at m/z 319.0 (base peak), suggesting the loss of glucose and myricetin as aglycon. UV data (λ_{max} 254, 265 sh, and 356 nm) was consistent with a glycosilation at the C-3 carbon of flavonols. A product ion at m/z 481.0 (myricetin-glucose) in the $[M + H]^+$ spectrum indicated that a rhamnose residue is in terminal position in the disaccharide moiety, while a glucose residue is directly linked to the aglycon. Compound **2** was thus characterized as myricetin-3-*O*-rhamnosyl-glucoside. Compound **3** (Table 2) exhibited a protonated molecular ion at m/z 435.0 $(C_{20}H_{19}O_{11})^+$, and the MS/MS spectrum in the positive ion mode showed a fragment at m/z 303.0, suggesting the loss of a pentosyl moiety attached to quercetin. Compound **3** was characterized as a quercetin-3-*O*-pentoside, probably quercetin-3-*O*-arabinoside.

Compound **4** (Table 2) exhibited a protonated molecular ion at m/z 641.2 $(C_{28}H_{33}O_{17})^+$. The MS/MS spectrum in the positive ion mode showed fragments at m/z 479.0 (60%) and 317.0 (100%), suggesting the successive losses of two hexosyl moieties and isorhamnetin as aglycon. Compound **4** is suggested to correspond to isorhamnetin-3-*O*-diglucoside. Compound **5** (Table 2) exhibited a protonated molecular ion at m/z 595.0 $(C_{27}H_{31}O_{15})^+$. The MS/MS spectrum in the positive ion mode showed fragments at m/z 449.0 (70%) and 287.0 (base peak), suggesting successive losses of rhamnosyl and hexosyl moieties, respectively, and kaempferol as aglycon. Compound **5** is suggested to be kaempferol-3-*O*-rhamnosyl-glucoside.

Compound **8** (Table 2) exhibited a protonated molecular ion at m/z 625.2 $(C_{28}H_{33}O_{16})^+$. MS/MS spectrum in the positive ion mode exhibited fragment ions at m/z 479.0 (50%) and 317.0 (base peak), suggesting losses of rhamnosyl and glucosyl moieties. Based on a product ion at m/z 479.0 (isorhamnetin-glucose) in the $[M + H]^+$ spectrum, it was inferred that the rhamnose residue is at the terminal position of the disaccharide moiety, while the glucose residue is directly linked to the aglycon.[23,24] Compound **8** was characterized as isorhamnetin-3-*O*-rhamnosyl-glucoside. Compound **6** (Table 2) exhibited a protonated molecular ion at m/z 771.2 $(C_{34}H_{43}O_{20})^+$, probably corresponding to a triglycoside derivative of **8**, containing a further rhamnosyl moiety. The MS/MS spectrum in the positive ion mode exhibited a fragment ion at m/z 625.1 (90%), suggesting the loss of a rhamnosyl moiety, while fragment ions at m/z 479.1 (50%) and m/z 317.1 (100%) suggested further losses of rhamnosyl and then hexosyl moieties, isorhamnetin standing as the aglycon. Compound **6** was tentatively characterized as isorhamnetin-3-*O*-dirhamnosyl-glucoside. Compound **7** (Table 2) was identified as rutin, by direct comparison with a standard sample analyzed in the same chromatographic conditions. Compound **9** (Table 2) exhibited a protonated molecular ion at m/z 449.0 $(C_{21}H_{21}O_{11})^+$. The MS/MS spectrum in the positive ion mode exhibited a fragment ion at m/z 287.0 (100%), consistent with the loss of a hexose moiety and kaempferol as aglycon; compound **9** is suggested to be kaemperol-3-*O*-glucoside.

Compound **10** (Table 2) showed a protonated molecular ion at m/z 641.0 $(C_{28}H_{33}O_{17})^+$, thus an isomer of **4**. The MS/MS spectrum in the positive ion mode exhibited fragment ions at m/z 495.0 (40%), suggesting the loss of a rhamnosyl moiety, and m/z 333.0 (100%), corresponding to patuletin as aglycon. Compound **10** was tentatively characterized as patuletin-3-*O*-rhamnosyl-glucoside.

Compound **11** (Table 2) yielded a protonated molecular ion at m/z 449.2 $(C_{21}H_{21}O_{11})^+$, consistent with quercitrin, a monoglycoside containing quercetin and rhamnose, which are moieties often detected in other constituents of samples C1−C7.

Compound **12** (Table 2) showed a UV broad absorption band at 380 nm and a shoulder at 250.0 nm, characteristics of a chalcone with hydroxyl group at position 4 of ring B and a protonated molecular ion at m/z 381.4 (Table 2). The MS/MS spectrum in the positive ion mode exhibited a fragment ion at m/z 147.2 (40%), probably corresponding to the shikimate derived C_6C_3 moiety, and indicates one hydroxyl group on ring B. The other fragment ion at m/z 235.2 (100%), corresponding to ring A, indicates a substituent with a carbon skeleton in addition to the hydroxyl groups commonly found on chalcone ring A. The available data do not allow the identification of the chalcone. Compound **13** (Table 2) exhibited a protonated molecular ion at m/z 761.3 and UV bands at 255 and 350 nm (Table 2). The MS/MS spectrum in the positive ion mode exhibited a base peak at m/z 615.3, suggesting the loss of rhamnose (146 amu), and a fragment at m/z 287.1 (40%), indicating kaempferol as aglycon. It is suggested that compound **13** is a kaempferol glycoside, containing rhamnose (146 amu), glucuronyl (176 amu) and galloyl (152 amu)[25] moieties, being tentatively characterized as kaempferol-7-*O*-rhamnosyl-3-*O*-galloyl glucuronide.

Compound **14** (Table 2) contains a hydroxycinnamic acid moiety, responsible for the absorption at 300−325 nm[11,16] and exhibited a protonated molecular ion at m/z 584.3 $(C_{34}H_{38}N_3O_6)^+$ (Table 2). The MS/MS spectrum in the positive ion mode focused at m/z 584.3 yielded a fragment at m/z 438.2 (100%), suggesting the loss of a coumaroyl residue (146 amu), and at m/z 420.2 (70%), indicating loss of water. Comparison of UV and MS/MS data with literature[11] indicated the presence of a coumaroylspermidine derivative that was characterized as *N*′,*N*″,*N*‴-tris-*p*-coumaroylspermidine (**14**). Compound **15** (Table 2) showed UV absorption band and fragmentation patterns in the positive ion mode similar to those of compound **14**, exhibiting a protonated molecular ion at m/z 674.3 $(C_{37}H_{44}N_3O_9)^+$, thus 90 amu higher than the protonated molecular ion of **14**. The MS/MS spectrum exhibited a fragment at m/z 498.2 (70%), suggesting the replacement of coumaroyl by feruloyl (176 amu) in **15**, and another fragment at m/z 480.3 (100%), suggesting the loss of water. The higher retention time of **15**, in comparison with **14**, is coherent with its lower polarity, due to the addition of a methoxyl group (present in the feruloyl moiety) as substituent.[26] Compound **15** was characterized as *N*′,*N*″,*N*‴-tris-*p*-feruloylspermidine by comparison of MS/MS data reported in the literature.[10]

Other authors have also reported the presence of flavonoids in pollen, particularly apigenin, quercetin, isorhamnetin, luteolin and genistein derivatives.[6,7] It is known that these compounds have a broad spectrum of biological activity.[21] Chalcone and hydroxycinnamic acid amides have also been reported in bee pollen.[14] These constituents have shown antioxidant activity, in both *in vitro* and *in vivo* biological systems.[8,14] Flavonol glycosides, such as quercetin, kaempferol and isorhamnetin derivatives, have been reported as major constituents of honey.[21] Flavonoids are normally found as glycosides in pollen. For example, pollen from *Echium plantagineum* contains glycosides of quercetin, kaempferol and isorhamnetin.[27] But flavonoids of pollen from *Eucalyptus globulus* are free aglycons. Eucalyptus honey also is poor in flavonoid glycosides and rich in aglycons.

Table 3. Distribution of Compounds 1−15[a] in Untreated, Frozen at −18 °C or Frozen and Then Dried Methanol Extracts of Pollen Samples C1−C7[b]

	substances														
treatment	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
C1															
control	+	tr[c]	tr	+	+	tr	tr	+	tr	tr	tr	+	tr	+	+
frozen	+	tr	tr	+	+	tr	tr	+	tr	tr	tr	+	tr	+	+
frozen/dried	+	tr	tr	+	+	tr	tr	+	tr	tr	tr	+	tr	+	+
C2															
control	+	tr	tr	+	+	tr	tr	+	tr	tr	tr	+	tr	+	+
frozen	+	tr	tr	+	+	tr	tr	+	tr	tr	tr	+	tr	+	+
frozen/dried	+	tr	tr	+	+	tr	tr	+	tr	tr	tr	+	tr	+	+
C3															
control	+	tr	tr	+	+	tr	tr	+	tr	tr	tr	+	tr	+	+
frozen	+	tr	tr	+	+	tr	tr	+	tr	tr	tr	+	tr	+	+
frozen/dried	+	tr	tr	+	+	tr	tr	+	tr	tr	tr	+	tr	+	+
C4															
control	+	tr	tr	+	+	tr	tr	tr	tr	tr	tr	tr	tr	+	+
frozen	+	tr	tr	+	+	tr	tr	tr	tr	tr	tr	tr	tr	+	+
frozen/dried	+	tr	tr	+	+	tr	tr	tr	tr	tr	tr	tr	tr	+	+
C5															
control	+	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	+	+	+
frozen	+	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	+	+	+
frozen/dried	+	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	+	+	+
C6															
control	+	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	+	+	+
frozen	+	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	+	+	+
frozen/dried	+	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	+	+	+
C7															
control	+	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	+	+	+
frozen	+	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	+	+	+
frozen/dried	+	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	+	+	+

[a] See Table 2. [b] See Table 1. [c] Trace amounts.

Eucalyptus nectar contains flavonoid sophorosides, but they are hydrolyzed by glucosidases from the bee saliva.[15]

N′,N″,N‴-Tris-*p*-coumaroylspermidine appreciably inhibits the HIV-1 protease. In addition, it was shown to have high activity against *Helicobacter pylori*.[11] Phenylpropanoid conjugates, including *N′,N″,N‴*-tris-feruloylspermidine, are predominant compounds from flowers and surfaces of pollen grains of *Arabidopsis thaliana*.[10] Pyrrole ketohexoside derivatives, named pollenopyrroside, were found in bee-collected *Brassica campestris* pollen.[28] Pyrrolizidine alkaloids have also been detected in bee pollen.[29]

The phenolic composition of C1−C7 varied, comparing one sample with another (Table 3, Figure 1). Some compounds are important constituents of all samples analyzed. Such are the cases of **1**, **14** and **15** (Table 3, Figure 1). Other substances are relevant constituents of some samples and trace components in others. For example, **4** and **5** are relevant components of extracts of samples C1−C4 (Table 3, Figure 1); **8** and **12**, of samples C1−C3; **13**, of C5−C7 (Table 3). Compound **8** (isorhamnetin-3-*O*-rhamnosyl-glucoside) is a major compound of sample C1 (Figure 1). Compounds **2**, **3**, **6**, 7 and **9**-**11**, all flavonoids, are seemingly minor constituents of all samples analyzed (Table 3).

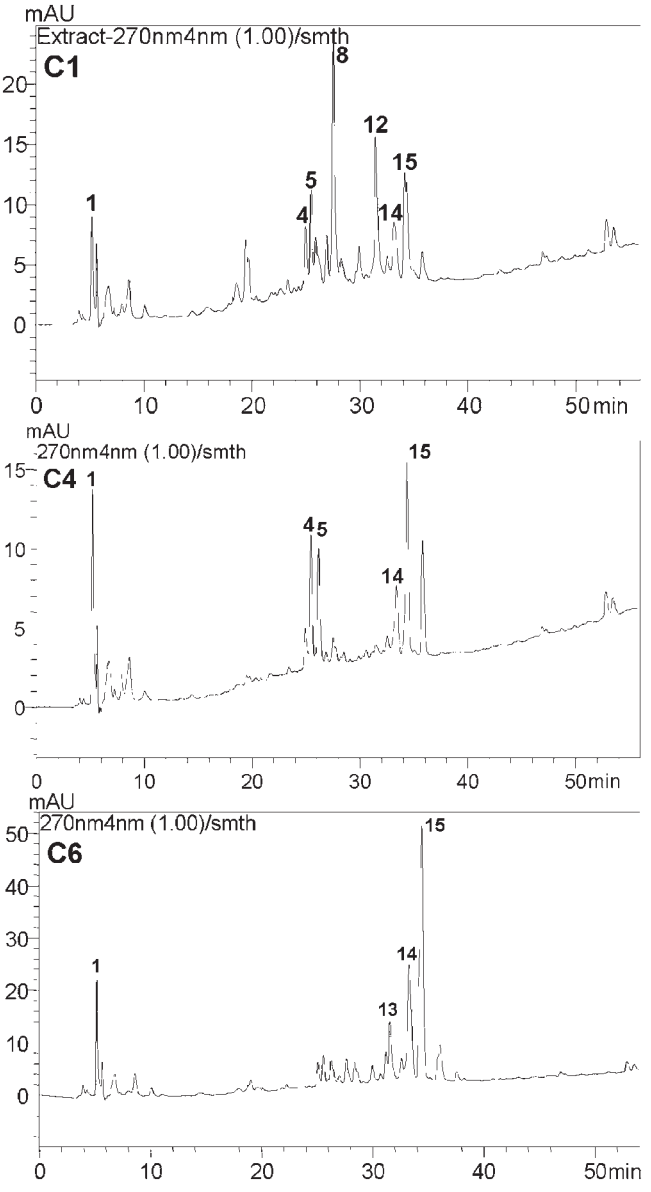

Figure 1. HPLC/PAD chromatograms of methanol extracts of bee pollen samples. See Table 1 for palynological composition of the pollen samples C1, C4 and C6 and Table 2 for names of substances corresponding to the band numbers.

While the rosmarinic acid derivative (**1**) and the spermidines (**14** and **15**) are important constituents of all samples analyzed, the flavonoids are selective, and may be major components of only some sample pollen extracts. The distinct phenolic profiles are certainly accounted for in the distinct pollen composition of C1−C7 (Table 1). However, the data of the present work do not allow tracing a correlating of the chemical profile with the palynological composition. For example, bee pollen samples C1−C3 have similar chemical composition (Table 3), but quite distinct plant pollen composition (Table 1). The results of the present work suggest that spermidines **14** and **15** (based on coumaric and ferulic acids, respectively) are common pollen constituents, irrespective of plant sources. Further chemical analyses are required to establish how frequent and abundant spermidines are among other pollen constituents.

Table 4. Total Phenolic Substances (Percent of Gallic Acid Equivalents on Pollen Dry Basis) and Antioxidant Activity (Percent of Discoloration of DPPH) of 25 μg/mL Methanol Extracts of Pollen Samples C1−C7[a,b]

	C1	C2	C3	C4	C5	C6	C7	MEAN
total phenols	2.1 ± 0.03 A	2.3 ± 0.03 B	2.2 ± 0.03 A,B	2.0 ± 0.03 C	1.7 + 0.03 D	2.1 + 0.03 A,C	1.6 + 0.03 E	
antioxidant activity								
untreated	86.4 ± 0.3 A,a	86.9 ± 0.3 A,B,a	88.3 ± 0.3 C,D,a,b	87.7 ± 0.3 A,C,a	75.9 ± 0.3 F,a	90.2 ± 0.3 E,a	89.0 ± 0.3 D,E,a	86.3 a
frozen	86.2 ± 0.3 A,a	85.5 ± 0.3 A,a	86.7 ± 0.3 A,a	90.4 ± 0.3 B,b	90.38 ± 0.33 B,b	91.3 ± 0.3 B,a	75.9 ± 0.3 F,b	87.1 b
frozen/dried	89.9 ± 0.3 A,B,b	92.5 ± 0.3 C,D,b	89.4 ± 0.3 A,b	93.8 ± 0.3 C,E,c	94.1 ± 0.3 E,b	91.6 ± 0.3 B,C,a	89.8 ± 0.3 A,a	91.7 c

[a] Table 1. [b] For antioxidant activity, samples untreated, frozen at −18 °C or frozen and then dried were considered. Values are expressed as mean ± standard error from triplicate analyses. Values with uppercase letters in the same line and lowercase letters in the same column indicate significant differences at 5%. A 90.1% activity was obtained with a 5 μg/mL BHT solution.

No differences were noted in the chromatographic analysis regarding the freezing and drying treatments of the C1−C7 pollen samples (Table 3).

Antioxidant Activity and Total Phenolic Contents. Total phenolic substances were determined by a modified Folin−Ciocalteau reagent method.[16] The results are expressed as gallic acid equivalents (Table 4). Phenolic compounds may contribute directly to antioxidant action, because of their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers. The basic mechanism of Folin−Ciocalteau method is an oxidation/reduction interaction promoted by phenols and other non-phenolic reducing agents.[16] It is known, however, that non-phenolic reducing compounds interfere in the Folin−Ciocalteau method. Some known interfering compounds are organic acids, sugars and amino acids. In addition, phenolics respond differently to the Folin−Ciocalteau reagent. In spite of these criticisms, the methodology continues to be widely used for determination of phenolic content in foods and beverages.[16,17,20]

Interest in natural sources of antioxidant molecules in food, beverages and cosmetics has resulted in large numbers of research papers.[30] Bee pollen has been shown to decrease the level of lipid oxidation products in erythrocytes, indicating antioxidant capacity. Among substances with antioxidant activity found in bee pollen stand out flavonoids and other phenolic compounds.[3,5] Several pharmacological effects of flavonoids and phenolic acids may be accounted for their ability to scavenge free radicals.[8,9,12,16] Free radical scavenging effectiveness of bee pollen is due mainly to phenolic constituents.[17]

Bee pollen collected from different areas or in different seasons may exhibit distinct antioxidant activity.[17] The pollen samples analyzed in the present work were shown to have considerable diversity of flavonoids and high contents of phenylpropanoid spermidines (Figure 1). From the chemical composition of C1−C7 (Tables 2 and 3), high antioxidant activity is expected. Results shown in Table 4 are coherent with this expectation. Methanol extracts from all samples were highly active, with no substantial differences among them. The antioxidant activity observed for samples C1−C7 was comparable to the activity of BHT solution used as positive control (Table 4). The pollen samples C1−C7 contain substantial amounts of total phenols (approximately 2% on dry basis). Results of Table 4 did not indicate correlation between the amounts of total phenols and antioxidant activity. Samples C5 and C7 have relatively low phenolic contents, but exhibited antioxidant activities comparable with samples richer in total phenols (Table 4). Relationships between structure and antioxidant activity are complex. In addition, association among phenolic compounds also affects overall antioxidant activity.[31,20] Thus, qualitative traits may be more important to determine the observed antioxidant effect than the total content of phenols. In flavonoids, it has been shown that the substitution pattern of the B ring and presence of 2,3-unsaturation and the 4-oxo group are important factors determining antioxidant activity.[32] While glycosylation reduces activity, *O*-dihydroxyls at 3′,4′ have the opposite effect.[30] Thus quercetin, with a 3′,4′-dihydroxyl system, is highly antioxidant. In addition to quercetin, samples C1−C7 have other flavonoids with the same or similar B ring substitution pattern: myricetin and patuletin derivatives (Tables 2). Although with no *O*-dihydroxyl system, derivatives of kaempferol and isorhamnetin probably contributed also to the observed antioxidant activities of C1−C7 methanol extracts. The spermidines **14** and **15** are phenylpropanoid derivatives. Antioxidant activity of hydroxycinnamic acid derivatives (for example chlorogenic acids) has been widely recognized.[33]

As can be seen in Table 3, the treatments of the pollen samples C1−C7 of freezing and freezing and then drying apparently did not change their chemical composition. However, the treatments increased significantly the antioxidant activities of the samples analyzed, particularly the treatment of freezing and drying (Table 4). The explanation for this result seems to be the reduction of the moisture content and the resulting concentration of active substances.

The distinct phenolic profiles, as seen in Figure 1 and Table 3, point to the possibility of using analysis by HPLC for characterizing pollen samples from different origins. Chemical and palynological analyses could converge to a feasible means for characterization of bee pollen samples. However, the present results should be viewed as preliminary, since neither was sufficient sampling available nor were statistical analyses carried out to evaluate this possibility.

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